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by Tumour-Stroma Interactions; A Microarray View of Cancer
Microenvironment

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13. ABSTRACT (Maximum 200 Words) <p>The focus: Despite compelling cell biological studies and histopathological observations incriminating stromal cells in tumorigenesis, our knowledge of the genes that mediate changes in the tumor microenvironment and interactions among various cell types in epithelial cancer and their role in tumorigenesis is limited. Here, we describe a comprehensive molecular characterization of stromal-epithelial cell interactions, using microarray analysis of co-cultured cell pairs. We further show that these gene expression changes indeed are common feature of in vivo profiles of invasive epithelial cancers.</p> <p>We provide proof of principle evidence that the in vitro system recapitulates some aspects of cancer cell biology and may contribute new markers to the invasive events. We directly demonstrate that events in the interaction of epithelial cells and their neighboring fibroblasts play initial role in recruiting inflammatory cells and provoking immune surveillance.</p> <p>A reverse signal transduction approach: Perhaps the biggest innovation lies in the demonstrated ability to discern cytokine molecules that provoke the gene expression in the co-culture. This in turn provides new working hypotheses to test in vivo, on the etiological role of particular molecules in dictating the clinical course of the disease.</p>				
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OC030008**Report (according to Statement of Work) of: "the Regulation of leukocyte infiltration into ovarian cancer by tumour-stroma interactions; a microarray view of cancer microenvironment".****Introduction:**

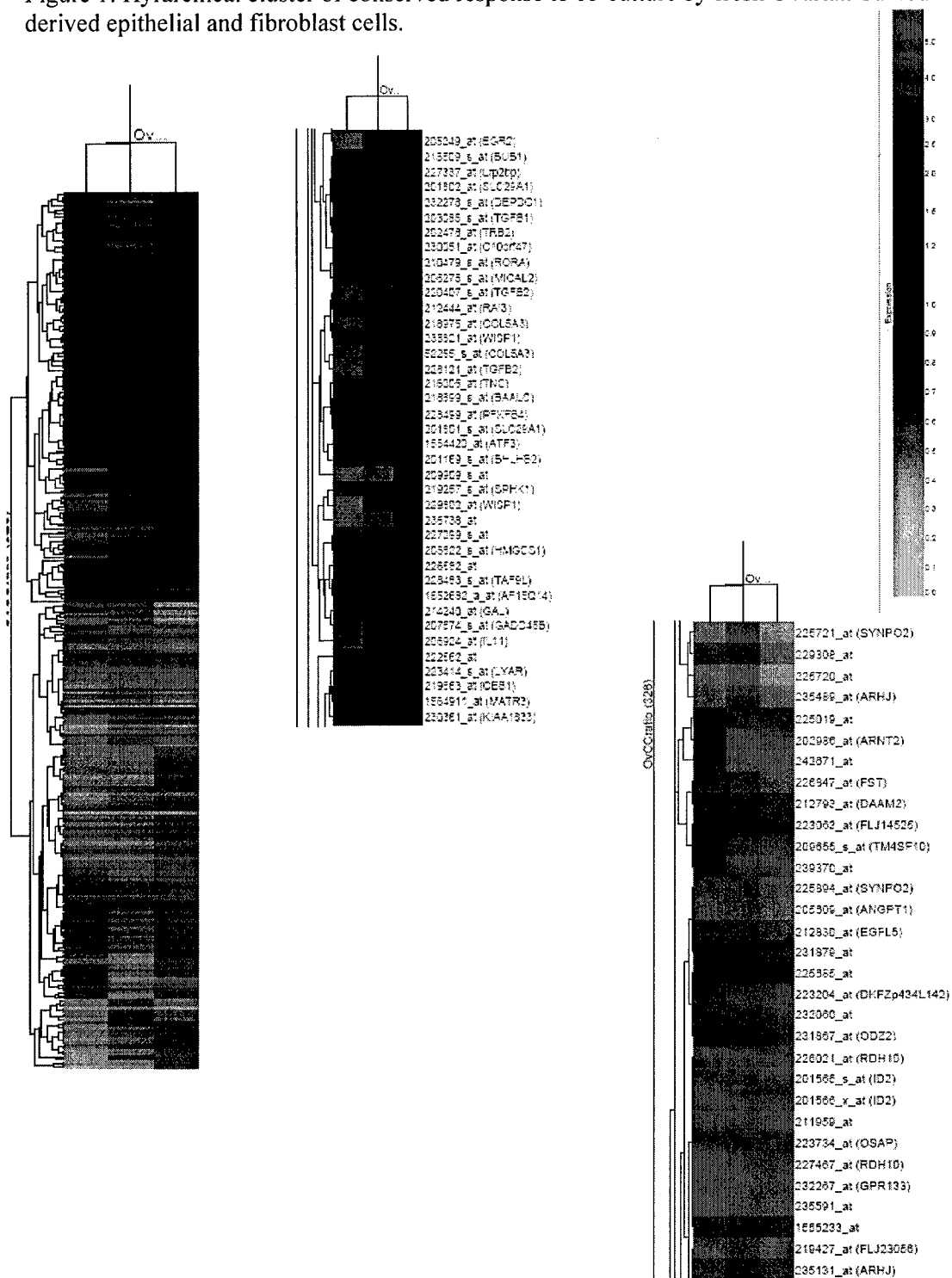
While it was known that characteristic alterations in the stroma, termed desmoplasia[1], occasionally accompany the formation of tumors[5-9], it was generally believed that these changes were simply induced by the tumor cells[10-13]. This model fails to explain why, when transformed tumor derived cells are placed in normal embryonic stroma [14, 15], or in normal extracellular matrix[16, 17], they regain normal phenotype. Moreover, recent results suggest that those changes may in fact precede the malignant conversion of epithelial cells [18-22]. Long-overlooked studies from the 1950s reported increased tumor formation after carcinogentreated stroma was transplanted with untreated skin epithelial cells[18, 23-25]. Cunha and colleagues demonstrated that the *in vivo* combination of normal human prostatic epithelial cells with cancer-associated fibroblasts (CAFs) led to limited tumor growth that resembled prostatic intraepithelial neoplasia, and that grafting CAFs with immortalized prostatic epithelial cells that were non-tumorigenic but expressed the SV40T antigen resulted in the formation of malignant tumors[20, 26]. So, oncogenic signals from the CAFs seem to stimulate the progression of a non-tumorigenic population of epithelial cells to a tumorigenic one. This model, if true, assigns a key role to the neighboring stroma in cancer initiation. However, before we target the stroma in cancer preventive treatment, we need a clearer definition of the stromal changes that ostensibly initiate epithelial cancers. This project uses microarrays as investigative tool to delineate cross talk between cancer cells and fibroblastic and immune components of stroma in ovarian cancer.

Task 1. To Extend an analysis of co-culture regulated genes in other appropriate cell combinations, including cells derived from different ovarian tumor stroma and epithelium (Months 1-6):

- a. **Prepare cells from different tumors with defined histology and use MACS separator and EpCAM-linked magnetic beads to separate the cells. Success is assessed on flow cytometry using 2G3, Laminin, FAP α and CK7 markers. On the array level extend the arrays from 10.5 k cDNA arrays to 19k oligoarrays (Compugen).**

We have so far isolated five cell cultures separated from ovarian cancers, that are >90% enriched for CK7 in the epithelial component, and >80% FAP α for the non-epithelial component. At this moment, however, we have not got enough of these cells to perform the array experiment. We are currently immortalizing (papilloma E6/E7) a fraction of these cells, so we can compare the immortalized and primary cells respond the same way to co-culture, so we can standardize an optimized method. In addition, we have characterized the response to co-culture on Affymetrix U133+2 full genome arrays, for three of these culture preps (Figure 1).

Figure 1: Hyrarchical cluster of conserved response to co-culture by fresh Ovarian Tumour-derived epithelial and fibroblast cells.



Whereas the gene expression signature identified by the initial experiments with OvCa cell lines was observed with one of the three fresh ovarian co-cultures, a canonical activation of TGF β signalling on the expense of ID2, ARNT2, and FST (typically counter-regulated), was conserved in all fresh cultures tested. In addition, we performed a large scale meta-analysis of the co-culture response signature across 1000 carcinoma arrays, ~200 of which are ovarian cancers, to identify genes that are most correlated with co-culture (see below). Genes overlapping between the affymetrix® arrays and the meta-analysis, were selected as TaqMan® probes for low density real time PCR array platform of ABI®, which follows 95 co-culture responsive genes, together with additional 30 genes which were used for Sybr-Green based real time PCR assays, were adopted as substitute to arrays as the monitor for the co-culture responses. This change in approach reduced expenses significantly.

To assess the conservation of our gene expression response, 15 Ovarian cancer cell lines were analyzed by co-culture experiments with foreskin fibroblast culture, by following 125 genes extended from the original experiment, using real time PCR (Figure 2). Twenty seven genes were scored by both primer sets designed in house, using SYBR-green as well as TaqMan™ primer sets from ABI®. The data is represented as

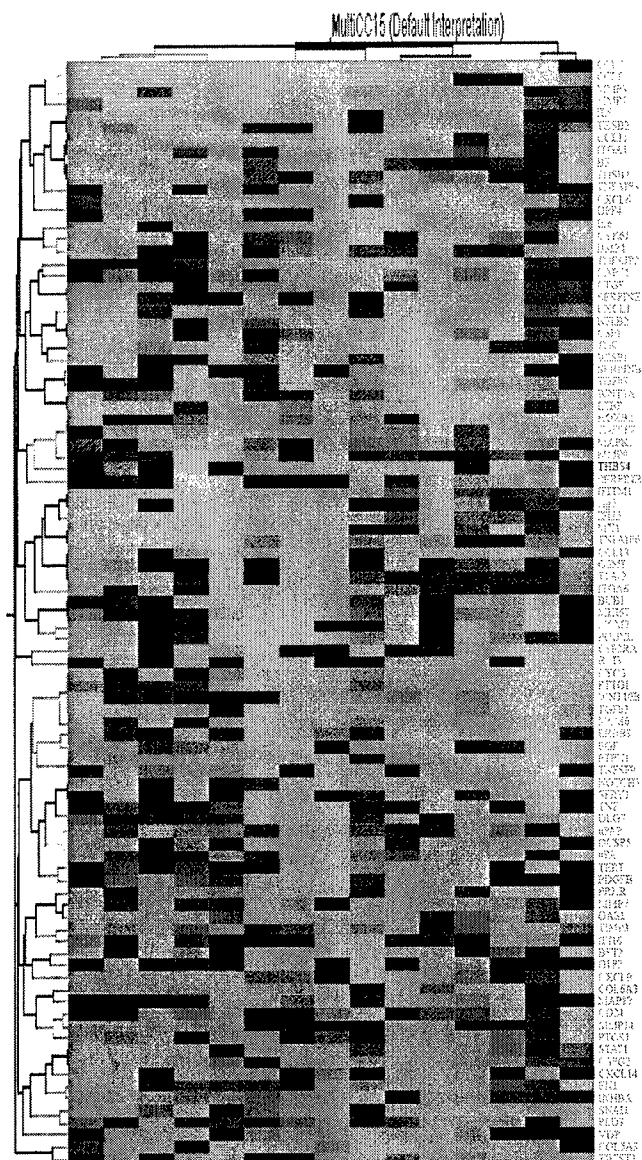


Figure 2. Co-culture versus isolated cells for 15 independent Ovarian Cancer cell lines. Genes are labelled according to how they were chosen; red is the original co-culture signature, blue is correlated genes from 765 individual cancer profiles from 24 tissues of origin, using Gene Recommender algorithm [2], green indicates genes published by others[3, 4].

$2^{\Delta\Delta Ct}$ of the gene against an average of 18S rRNA, GAPDH, and a control gene which is least changing in our system (from the microarray results). The independent primer sets showed average correlation of $R=0.85$ with each other and $R=0.71$ with the array results. Seventeen genes, IL6, IL8, CCL7, CCL8, MCM6, MMP1, MMP3, PRKR, PTPN1, CCL11, COLV α 3, CXCL1, CXCL6, CYCS, TGF β 2, TNFAIP2 and CSPG2 showed above 2 fold change in more than 10 cell pairs. For those genes, we found a similar response in co-cultures with three breast epithelial cell lines, one gastric cancer epithelial cell line, four ovarian theca fibroblast cell lines, three foreskin fibroblast cultures, and one lung fibroblast cell line (not shown). By contrast, most genes split into two signatures, each induced in more than half the cell pairs (Coherent red bar group on the left, and more diverse group on the right). Within each of the two signatures, some cell lines, such as A2780 and OVCAR3, exhibited little response. **Collectively, these findings demonstrate that it is possible to identify genes that are specifically induced *in vitro*, in response to cross-talk between fibroblast and epithelial cells, provided cell density and count in each culture are controlled.** The repeats of the experiment highlight genes that are more universally induced in co-culture. Also, this information allows to define the number of distinct responses that can use subsets of these genes.

To assess the conservation of our gene expression response, 15 Ovarian cancer cell lines were analyzed by co-culture experiments with foreskin fibroblast culture, by following 125 genes extended from the original experiment, using real time PCR (Figure 2). Twenty seven genes were scored by both primer sets designed in house, using SYBR-green as well as TaqMan™ primer sets from ABI®. The data is represented as $2^{\Delta\Delta Ct}$ of the gene against an average of 18S rRNA, GAPDH, and a control gene which is least changing in our system (from the microarray results). The independent primer sets showed average correlation of $R=0.85$ with each other and $R=0.71$ with the array results. Seventeen genes, IL6, IL8, CCL7, CCL8, MCM6, MMP1, MMP3, PRKR, PTPN1, CCL11, COLV α 3, CXCL1, CXCL6, CYCS, TGF β 2, TNFAIP2 and CSPG2 showed above 2 fold change in more than 10 cell pairs. For those genes, we found a similar response in co-cultures with three breast epithelial cell lines, one gastric cancer epithelial cell line, four ovarian theca fibroblast cell lines, three foreskin fibroblast cultures, and one lung fibroblast cell line (not shown). By contrast, most genes split into two signatures, each induced in more than half the cell pairs (Coherent red bar group on the left, and more diverse group on the right).

- b. Express ectopic CDH1 in OvCa lines with mesenchymal morphology (Hey and A2780), or pSUPER-based siRNA knock down vectors for this gene in epithelial-like OvCa lines (such as OVCAR-3). Use parental and derived cells in co-culture experiments. Score for gene expression changes by RT-PCR (Months 2-6).

Interestingly, a common reaction to the co-culture (like 2008), involved induced expression of SNAI1 and SNAI2, or TWIST1. This implies that the interaction with the theca cells induces EMT in epithelial-like ovarian cancer cell lines. Similar results were observed with the fresh cultures. Activin A receptor 2, JAK1 and slit2 were elevated in the non-reactive lines, while STAT3 was elevated in the reactive lines. These genes were still suggestive of cell adhesion and polarity as playing a role, in a similar manner as proposed in the application, but not necessarily through CDH1. We therefore explored the meta-analysis for gene correlated with the co-culture genes, using the gene recommender algorithm[2], and three existing multi carcinoma expression

profiling datasets[27-29]. The genes that were most correlated with the co-culture response (described further below) included SNAI2 and not SNAI1 or TWIST1. We therefore analysed the expression of these genes in 115 Ovarian carcinoma expression profiles conducted on Affymetrix U133+2 platform, with samples from the AOCS (OC00109). The SNAI2 gene was highly correlated with the co-culture response genes (Figure 3). The figure marks the genes that overlap between the group of genes correlated with SNAI2 (the gene cluster group) and co-culture (Genes in green or red). This suggests that the EMT modification of the co-culture should be addressed via SNAI2, rather than CDH1.

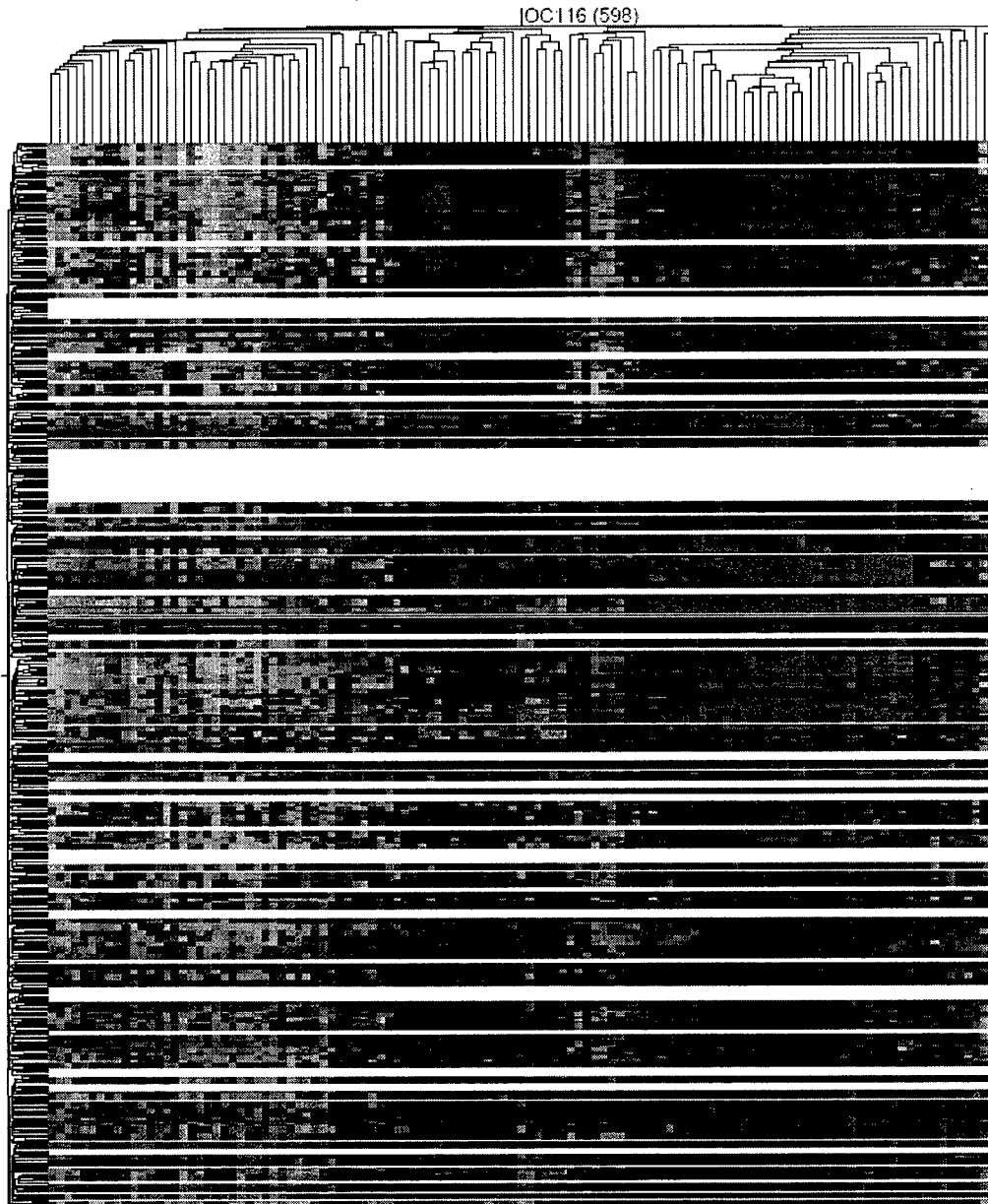


Figure 3: overlap between SNAI2 correlated genes across 116 OvCa expression profiles from the AOCS, and the co-culture genes.

The 513 genes overlap between 598 and 690 genes is highly significant ($P=0.0015$). Also, second most correlated to SNAI2 is CDH11. TCF8 represses CDH1 (E-cadherin) expression by directly interacting with the CDH1 promoter.

c. Similar to Task 1b, explore defined cellular changes to the ovarian epithelial cell line including DNA damage and anoikis, hormone responses, etc. (Months 6-9).

We tested the possible role of the found genes in modulating the response of the ovarian cancer cell lines to neighbouring fibroblasts, using cytokine treatment, such as Activin A or PDGF, and siRNA reagents directed against these genes. We repeated the co-culture experiment, in the presence of oestrogen, progestin, FSH, IL6, uPA, RGD peptides, and cisplatin (DDP). We did not find significant modification of the co-culture response in any of those. We intend to repeat these experiments, as the results were relatively surprising. Supporting this point, the genes that differentiate OvCa cells that provoke co-culture reaction and those OvCa cells that don't, contain neither component of such mechanisms such as DNA-damage proteins.

d. Identify potential new candidate soluble molecules as mediators of the stroma inflammation, in case the expression of their genes are affected by the EMT. Explore those factors as in Task 2. See task 2.

RNA interference of the key regulators of the SNAI2 expression and EMT transition, as depicted by the ovarian cancer expression profiles are sought from the new Elledge/Hannon library (Openbio®/GeneNet®, DoD funded resource and collaborative with us). These include WNT5A, CDH11, TCF8, ETV1, DDR2, RUNX1, TCF4, ID2 and ID3, PDGFRB and BMP4. The vectors are all available and with this strategy, much of the possible trouble with cloning and transfection are avoided. The cells that are transduced by these lentivirus vectors will be assessed in co-culture, and if their response is attenuated, the effect on co-transplantation with ovarian stroma to mouse xenografts will be compared with parental ovarian cancer cells. The most likely candidate soluble molecules come from the FGF and WNT family. This possibility is being explored by following SNAI2 and SPARC expression response to co-culture, after siRNA-mediated knock down of ATM, GSK3 β , FzD, and WNTs, as well as FGF7 and FGF8 (expressed in the ovarian stroma). Although this section of the project has not been performed in time with the SOW commitment, with the one described in the grant application, we did state in the application that we will use ovarian carcinoma expression profiling to focus our work on more likely physiologically relevant mechanisms and genes than the ones proposed. Such was the case with the EMT. We regret that by the time of report submission, we do not have the final results to share with the DoD reviewers.

e. Microarray profile the gene expression of cultures of non-fibroblast stromal cells, when grown in serum free medium versus conditioned media conditioned by epithelial or fibroblast cultures, as well as co-cultures thereof. Tested cells will be endothelial (HUVEC), Tumor Infiltrated Lymphocytes (TIL), macrophages, Theca albea, and Granulosa cells (Months 6-12). In case the soluble molecules appear labile, we will use tissue culture inserts (as in figure 5) rather than conditioned media.

We have profiled CD45+ TIL cells in the tripartite co-cultures, using transwell inserts. The RNA we produced in this manner were not with good enough quality for the Affymetrix arrays we are using now, U133+2. We are going to modify these to XP3 arrays which allow more amplification, and we will also unmask the Leukocyte mRNA from prevalent mRNA such as Globin, using GLOBINclear beads. Using RT-PCR we have however been able to detect Macrophage/dendritic cells increase in CXCL9, CCL18, CD68. We also detected Lymphocyte decrease in IL2R γ , IL4, CCL3 and CCL4 gene expression. Granulosa cells increase INHBA, GCSF, WNT5A, chemokines, and decrease expression of FST, StAR and RARA.

f. Define binary and ternary cell-cell interactions using the Task 1d. Construct a table of inductive gene expression related interactions among the different stroma components.

This task is being progressed in an ongoing fashion. We have used such a diagram in the preparation of a successful OC050265 grant this year. This diagram will continue to change as more results are generated. The hypotheses that such a diagram is generating are constantly checked against expression profiles from a constantly growing dataset of ovarian cancer expression profiles, as well as xenograft models of ovarian cancer tumour growth. We include a number of such co-transplanted ovarian cancer xenograft assays. These show that the effect of the fibroblasts is constantly increasing the cancer growth. All the cell pairs that increase in growth in response to co-transplantation, share a gene expression response to co-culture, which is similar to the fresh co-culture response, as shown in figure 1. Also integrated are expression profiles of the AOCs ovarian cancer patient specimens. These provide evidence for correlation between any response of the epithelial-fibroblast co-cultures and the infiltration in vivo of additional cell types listed in Task 1e (using their cell marker mRNA and expression correlation tools).

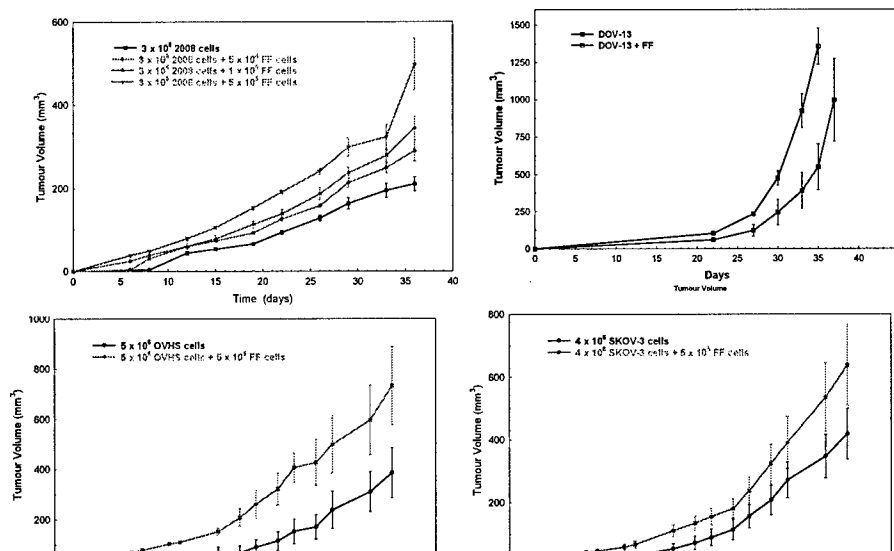


Figure 4: comparison ovarian cancer cell lines growth rate in xenograft between when human ovarian fibroblasts are included or not.

Task 2. To identify the molecule(s) that mediate the co-culture response.

- a. Assign responder-secretor role to the cells in the co-culture. For example in point a.2.7 epithelial cells is the secretor of $TNF\alpha$. The first interaction we will focus on is the response of the macrophages to a fibroblast-derived factor, that potentiates the macrophage to recruit T cells. Here the responder is the macrophage and the secretor is the inflamed fibroblast.**

We are now characterizing the response of mono-cultures of these leukocytes to these cytokines/chemokines. The combinations tested are mainly focused on chemokines/cell pairs that lead to migrational effects. We are currently troubleshooting the difficulty to attain good quality array results from the leukocyte population (with and without co-culture or in response to commercial chemokines/cytokine).

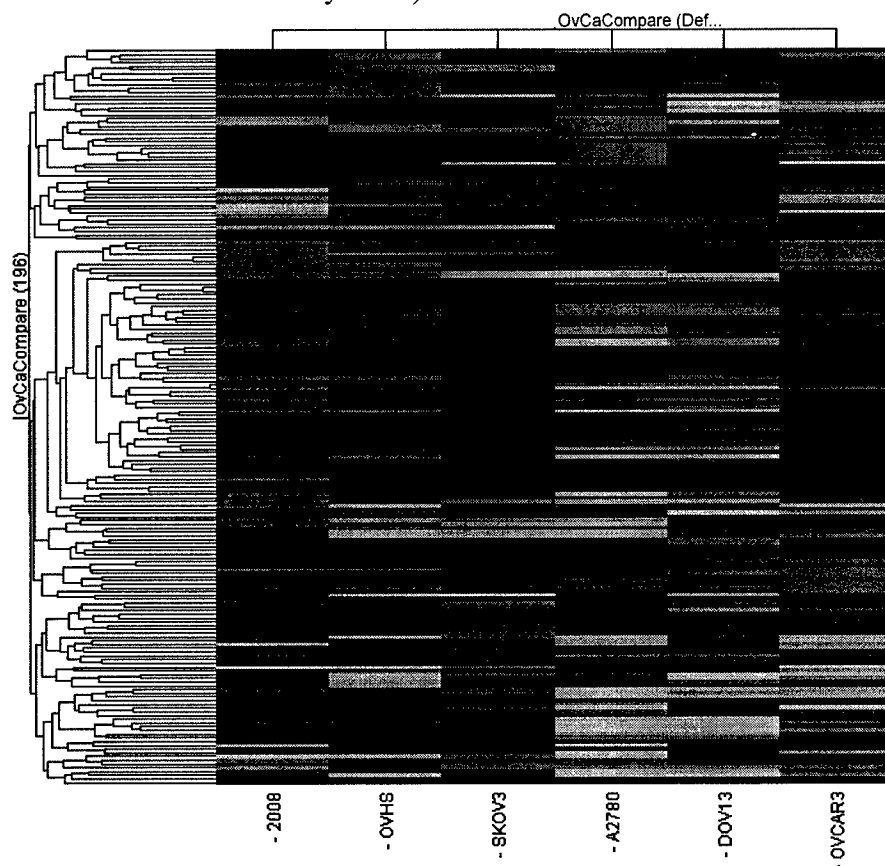


Figure 5: compare ovarian cancer cell lines grown in serum free media to identify secreted molecules that can explain the difference in co-culture responses, in order to identify candidates to try on cells. Cellular expression profiles are compared on Affymetrix U133+₂ platform.

b. Use array profiles to compare the secretor to the responder, looking for secreted molecules that are specifically overexpressed by the secretor. These will be considered as candidate for mediating the gene expression changes in the responder.

Affymetrix arrays were used to compare responsive ovarian cancer cells, non-responsive cells, and fibroblast cells for the purpose of selecting candidate mediators (Figure 5). In addition, we used Chemmyarrays (Figure 6B), to determine the appropriate candidate, and assign responder secretor role to cells (Task 2a). Of 120 cytokines and growth factors, we detected IL6, IL8, MCP1, CXCL1, TIMP-1, TIMP-2, EGF, and TNF α in these blots. We added Interferon- γ , IL1 β , IL6, TNF α , MCSF, GMCSF, TGF β 1, TGF β 3, bFGF, LIF, MCP1, IL8, IL18, Amphiregulin, Epiregulin, Oncostatin M, INHBA, INHBAB, CXCL1, CXCL3, CCL7, PDGF, and EGF and followed the response to these factors in normal human ovarian fibroblasts using quantitative real-time PCR. For reference, we included a number of paradigm co-culture responses.

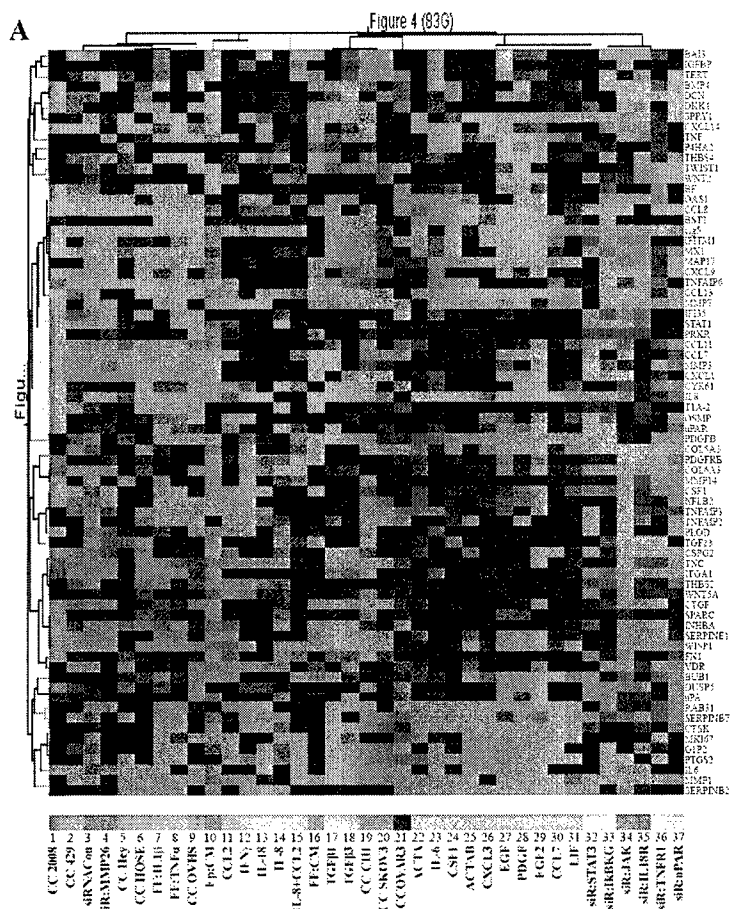
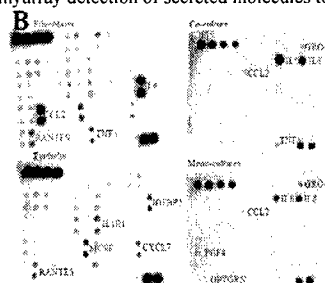


Figure 6: A. Identification of signaling molecules using RT-PCR based profiling of the response to candidates, and siRNA modification of co-culture reaction. Experimental color: Aqua=inflammatory co-culture, Purple=TGF β -like co-culture, Orange=Factor treatment of Fibroblasts, Green=Transwell, siRNA on 2008 cell Co-cultures Red=control genes, Yellow=Critical signaling genes. **B.** Chemarray detection of secreted molecules to choose candidates for **A**.



c. Apply candidate proteins (hopefully commercially available, otherwise express in transfected cells) on the responder cell monoculture (Months 6-9).

Using the candidate choices from Figure 5 and Figure 6B, we tested the expression response profile of fibroblasts to 23 distinct factors, and of epithelial cells to 16 distinct factors (Figure 6A). $\text{TNF}\alpha$ was still responsible for the most effective response in the fibroblast (columns 7, 8), for the biggest list of ovarian cells. This was further supported by siRNA-mediated attenuation of the response (Figure 6A, columns 32-37). Having generated an we used Genespring (version 7.2) to determine which cytokine treatment of mono-culture best mimics each co-culture. $\text{TNF}\alpha$ and $\text{TGF}\beta$ prevailed most of the responses we characterized. We still want to test WNT and NOTCH proteins.

d. Score for T-cell recruitment to macrophages, in response to the treatment. In parallel, monitor gene expression changes induced by these commercial factors by microarray (three slides for each factor). Compare the global gene expression changes that occur in response to the commercial factor to those that occur in co-culture or in response to 2 fibroblast conditioned media. Use LODS Bayesian plots of the array results, clustering and principle component analyses to look for maximal similarity.

We observed recruitment of CD45^+ and macrophages from either tumour mass or from Buffy coat blood donations, into the co-culture environment. As an initial step in identifying the molecules that modify the macrophage we tested the directional migration attraction of the macrophages and lymphocytes into mono and co-cultures of epithelial and stromal cells. We then accessed a panel of chemokines and cytokines that are expressed in the co-culture, and tested their ability to attract macrophages and lymphocytes as commercial recombinant proteins.

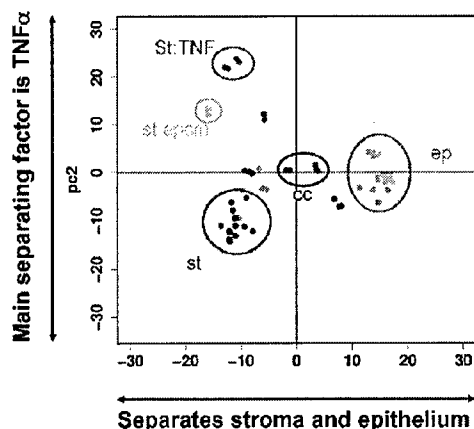


Figure 7: PCA analysis of different treatment of mono-cultures compared to the co-culture as proposed. $\text{TNF}\alpha$ appears as the most significant contributor to the co-culture response.

We tested FGF7, FGF2/bFGF, $\text{TGF}\beta 1-3$, CXCL12/SDF1, IL8, CCL2, CCL5, CCL7, CCL8, CXCL1, CXCL3, CXCL6, CXCL7, CXCL9, MCSF, IL11, IL6, OSM, IL1 β , IL18, ActivinAB, InibinA, Amphiregulin, HB-EGF, Epiregulin, EGF, FLT3LG, PDGF-AB. We found that bFGF, CCL7, CCL8, CCL11 and CXCL3 attracted CD45^+ cells in minutes. We used FACS to characterize the cell population that is being attracted. Macrophages, Eosinophils, MAST cells and T and NK Lymphocytes are attracted to different chemokines. Although at the time of the application we suggested that the macrophages are expressing the CXCL9 in the tumours, using the multi-co-culture experiment (Figure 2), shown us that more than 50% of the OvCa carcinoma cells will express CXCL9 by themselves in direct response to the co-

culture. We do however believe that macrophages are also recruited through CCL2, CCL7 and CCL8, as we have immunohistochemistry stains of CD68 in tumour juxtaposed position, much like $\text{FAP}\alpha$, TNC or SPARC.

- e. Acquire neutralizing antibodies against candidate molecule (that fulfilled Task 2b requirements). Apply these antibodies onto a co-culture experiment. Check if antibody diminishes the co-culture response (Month 10).**

As siRNA tools (Ambion® silencer and human (bar coded+95b) shRNA- lentivirus library latest version (Hannon/Elledge/OB product)) become more reliable and easy to obtain than neutralizing antibodies, Task 2e and 2f have switched in priorities. Downregulation is confirmed with ABI® TaqMan assays that are confirmed to target the same mRNA as Ambion's siRNAs. We first test siRNA short oligos in transient transfections, and then if they work (Figure 6A, columns 32-37), we obtain the equivalent sequence in invitrogen gateway lentivirus vectors. The lentivirus vector system expresses six independent shRNAs against the target locus/mRNA. It also contains a bar code oligo sequence that confirms the identity of the clone, and improved promoter system. If the RNA knock down reagents are effective, we obtain independent validation through neutralizing antibodies. We so far have obtained antibodies against TGFβ, IL1β, IL18, FAPα, MMP1, MMP2, MMP3, SPARC, and TNFα. The chemokines that are active in the epithelial-stromal interactions are not as reagent rich as the classical cytokines.

- f. Construct siRNA vectors (pRETROSUPER) for knockdown of candidate molecules (if ovarian epithelial cell is the "responder" in the co-culture, siRNA of the receptor for candidate molecule will be targeted) and transfect them into the ovarian epithelial cells, clone plasmid-carrying derivative cells. Validate that these vectors mediate expression change of their specific genes in stable ovarian epithelial cell clones (Months 7-10).**

We are in the process of obtaining and managing a clone set (similar to cDNA arrays) of the Elledge/Hannon genome-wide shRNA lentivirus library[30]. This will spare us the need to clone and construct the reagents against the target gene of interest. We so far have obtained seven siRNA molecules (screened from within 240 siRNA molecules) that modify the co-culture reaction in different ways and to different degrees, on different cell pairs. Ambion shares the sequence information of the effective clones with us, and we will also create our own shRNA equivalent of the same sequence, into Invitrogen® Gateway lentiviruses.

- g. Confirm the role of the candidate molecule in the co-culture. Perform co-culture microarrays on derived clones, compare to parental lines for co-culture response (Months 8-12).**

To further investigate the two main co-culture responses, we performed an un-supervised clustering using spearman-confidence (Figure 6A). One type of co-culture responses (columns 1-6) was adjacent to IL1β/TNFα (columns 7, 8). A second type of co-culture response (column 19-21) was adjacent to TGFβ treatments (columns 17, 18). Knock-down (siRNA-mediated) of control genes (Columns 3, 4), was indistinct from unmodified cells, while siRNA of TNFR1 (column 36) and NIK (IkBKG, column 33) in the fibroblasts, eliminated most of the response to co-culture. This highlighted CRGs that depend on TNFα (green bar on the right) or TGFβ (cyan bar). Simply using gene annotation would not produce the same result, as TNFAIP2, TNFAIP3 and NFκB2 were not affected, while MMP3 and TNFAIP6 were. Red bar on the right marks a partial list of CRGs that responded to multiple treatments, and a therefore not useful for identifying mediators of the signal exchanged.

- h. Repeat this analysis for epithelial response to fibroblast that appears like an interferon- γ response (fibroblast is secretor and ovarian epithelial cell is responder), as in Task 2a-e (second year).**
- i. If Task 1a-e identify new co-culture responses that are robust and hint at clinically relevant processes in the AOCS data, repeat this analysis for this new response, as in Task 2a-e (year 3).**

Analysis of the time course of co-culture found TNF α is induced in 8 hours of the co-culture. We therefore tested which cytokines induce TNF α expression in epithelial cells. We found EGF+IL8 or EGF+LIF induce a 200 fold increase in TNF α in the epithelial cells. Indeed, siRNA against IL8RA attenuates the co-culture (not shown). This observation is consistent with IL8 being expressed by the fibroblasts (Figure 6B).

Task 3. To compare the expression pattern of key molecules that come up in co-culture in surgical specimens from CDRP project OC000109, with the aim of assessing how general our observations are, and in order to relate our observations with *in vivo* clinical features such as lymphocyte infiltration in tumors and patient survival (parallel to Task 2, after each candidate is identified).

- a. Establish as many as possible Tissue arrays with needle punch sections arrayed on glass slides.**

150 of AOCS ovarian cancers and parallel tissue banks have been generated in Peter MacCallum Cancer Centre for this project.

- b. Obtain antibodies against candidate molecules that are reactive and specific in Immunohistochemistry.**

A drawback in the task is that many of the interesting genes are not reactive on formalin fixed material and require fresh frozen tissue. This means we stain individual specimen, rather than the high throughput of the tissue array. We are in the process of testing antibodies that recognize phosphoproteins that are part of the cascade of signal transduction pathways that we so far identified, such as TNF α and chemokines receptors. This will be used for double staining with CRG genes to correlate the signal transduction pathway activity with the expression of target genes that were upregulated in our co-culture experiments. So far, we correlated between a number of genes, which were identified as co-culture correlated in the cancer tissue expression arrays using the gene recommender, and the co-culture gene products in the protein level. This includes SFRAP4, Thy1, SPARC, uPA, uPAR, INHBA and PDGFRB. This serves as independent evidence for the validity of the correlations identified by the gene recommender.

- c. Stain for candidate molecules by both in situ hybridization for RNA expression, as well as immunohistochemistry to assess correlation of expression with biological processes that are the focus of this study, such as TIL (CD3+ cells in the tumor sections), angiogenesis, inflammatory reactions, invasion, and apoptosis.**

AOCS tissue arrays were stained for a panel of stromal cell type specific markers, including CD57 (NK), CD11 (macrophage), CD3 (T lymphocytes), CD4, CD8, CD19 (B cells), CD31 (endothelial cells), CD45 (leukocytes), CD24 and SCA1 (potentially stem cells), CD68 (monocytes), CK7 (epithelial cell), vimentin and α SMA (mesenchymal cells, EMT), as well as

some oncogenes and tumour suppressors, such as ER, PR, ErbB2, TP53, and activated CASP3. Ten CRG protein products are being stained on tissue microarrays currently, results are not available at this stage, apart of MMP2 and CD68 which are consistent with the analysis described in task 1d. These stains were used to correlate the specimens that express relatively elevated levels of the co-culture gene on the array expression profiles, with degree of immune cell infiltration, oncogenic status of the cancer cell, EMT, apoptosis, and angiogenesis.

d. Assess our hypotheses in light of the expression associations with the known clinical data of those specimens.

The Co-culture correlated gene list is highly overlapping ($P=10^{-7}$) with a signature that predicts outcome in ovarian cancers[31]. Figure 8 shows how co-culture response genes form a network of coexpressed genes in ovarian cancer, in a level of coordinate coexpression comparable to cell cycle control genes. Further, the specimen that present elevated expression in the co-culture-correlated genes (top node), and those with elevated expression of cell cycle genes (bottom node) are partially overlapping, and both or either cover most of the cancer cases. This suggests that these are redundant independent events that can each lead to cancer. By grouping the samples of the 94 ovarian cancer profiles, according to the expression level of these gene networks, we tested the correlation between the event mimicked in the *in vitro* co-culture, and patient survival. Figure 9 shows that the inflammatory, co-culture correlated genes are predictive of the disease free survival of the patients (bottom), while, the cell cycle genes are not (top).

116 Ovarian Cancer and LMP expression profiles

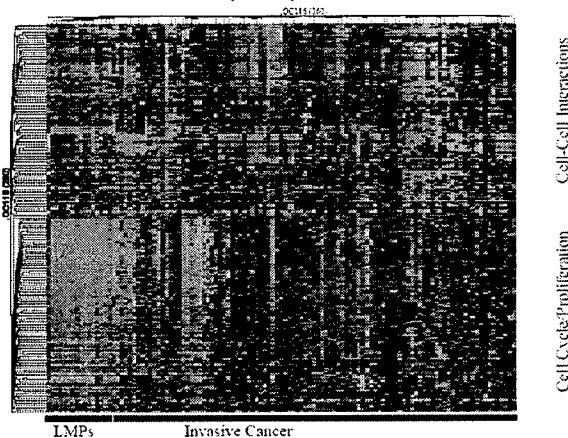


Figure 8. Top: Cluster distribution of 116 Ovarian Cancer profiles on Affymetrix U133+2 arrays (CDMRP00109), using both 130 genes that came up in the co-culture experiment (top node), as well as 130 genes that cells express during cell cycle and proliferation (bottom node).

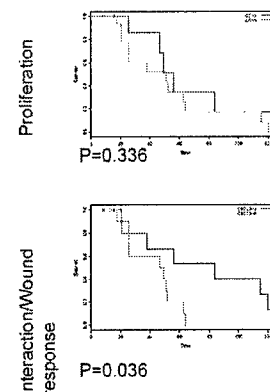


Figure 9: Outcome prediction in ovarian cancer patients of the AOCS patients when grouped according to expression of cell proliferation genes (top), or co-culture correlated genes (bottom). Associated P value is indicated.

This division of the ovarian cancer samples agrees with EMT and inflammatory infiltrate status of the same cases. This preliminary result, if it will persist, will call for a paradigm shift as for what features of the cancer biology are best to target for both therapeutic and diagnostic purposes. The results suggest that cancers either alternate or persist between two stages that are

at least independent and possibly partially mutually exclusive; growth and spread. To the best of our knowledge, the interesting link between EMT and immune cell infiltration is presented here for the first time (although chemokines have been suggested in a similar process[32, 33]).

Task 4. To characterize the biological significance of the gene expression changes observed in co-cultures and their effects on cancer progression and immune surveillance of cancers *in vitro* and *in vivo*.

- a. Use flow cytometry kits like APO-BrdUTM in CytoPerm-Cytofix™ buffers, for the analysis of cell cycle and cell death process in cells growing in monocultures versus co-cultures. Individual cell populations are analyzed in the mixed cell populations by gating the results with CK7 as marker for epithelial cells.**

This assay was performed a number of times, and in spite of some cell-cycle control genes showing elevated expression in the epithelial cell (such as MCM2 and MCM6), no significant change in cell cycle distribution of either cell type is observed. Either those genes have additional activity, or, these are not the rate limiting step for cell cycle progression for these cells.

- b. Construct Ectopic expression vectors for molecules that are found in Task 2 to confer the co-culture responses. Use a modified version of pBABE vectors, carrying ovarian 3 specific promoter.**

As for the shRNA vectors, we are in the process of building a genome-wide library of expression vectors for full length mRNAs. The clones of relevant genes will be used from the library to spare unnecessary cloning.

- c. Transfect obtained vector into epithelial cancer cell lines that are less active in the co-culture assays, such as A2780 (Months 7-10).**

Gene modified ovarian cancer cells are constantly being derived and tested for the co-culture response (as in Figure 6A).

- d. Construct knock down vectors siRNA vectors (pRETROSUPER vector), for the same genes as in task 4b.**

As mentioned above, we are going to use existing libraries from Open Biosystems and GeneNet®.

- e. Transfect those into epithelial cancer cell lines that are less active in the co-culture assays, such as 2008 or Hey.**

- f. Construct expression vectors for negative dominant genes such as SOCS to disrupt the interferon response in the epithelial cells.**

Such constructs for SOCS1 and SOCS3 were obtained from D. Hilton, and are being introduced into cells.

- g. Transfect epithelial cancer cell line A2780 and 2008 with these vectors.**
In progress.

- h. Perform co-culture microarrays on derived clones, compare to parental lines for co-culture response (Months 8-12).**

Evidently, this timeline statement was not meant for year 1.

- i. Choose cell derivatives (carrying a gene expression aberration for the molecules mediating the co-culture response), and characterize their malignant potential in mouse xenograft models.**

See task 1f. In progress.

- j. Assess the response to the transplant by the mouse stroma (compare gene-modified cells to parental).**
- k. Introduce human stromal components together with the ovarian cancer cell line. Start with fibroblasts, then macrophages.**

Task 1f.

- l. For Lymphocyte recruitment, ovarian epithelial cancer cells will be used from our own tissue bank collection. These cells have corresponding peripheral blood lymphocytes, which avoids allograft rejection reactions in the xenograft. Obtain these lymphocytes, co-inject them with the cancer cells, fibroblasts, and macrophages, all from the same patient (so far have such cell cultures from two patients).**

Five patient worth of such complementing cell collections have been obtained. We have not introduced these into mice, until we learn more how to optimize the effect of the fibroblasts on the OvCa growth (as in Task 1f).

- m. Follow mice for survival, tumor infiltration with lymphocytes, inflammatory reaction, cancer histology, tumor burden time course, immunohistochemistry with antibodies against co-culture induced gene products (such as CXCL9 and MMP2).**

Key Research Accomplishments:

- 1) Assessing physiological relevance and prevalence of the gene expression response observed in co-cultures of ovarian epithelial and fibroblast cells to ovarian cancer profiles.**
- 2) Dissecting the factor dialog that gives this response, and assessing the validity of the conclusion drawn from the in vitro system to human tumours.**
- 3) Identifying reagents that would be powerful tools for modifying the intercellular interactions specific to tumours and for further drug selections for this purpose.**
- 4) Formulating approaches to prioritize cell biology research at events which in ovarian cancer are more likely to control patient survival and clinical progress.**

Reportable Outcomes

- 1) The work reported here was the foundation for a successful idea development grant ("Identification of Ovarian Cancer Susceptibility Genes Involved In Stromal-Epithelial Interactions", OC050265, PI Chenevix-Trench, 5% time commitment from me,**

\$650K) with overall score 1.4 and full of complements, particularly commending the preliminary results we presented (essentially as in this report).

- 2) We are in the final steps of submitting this work to publication, with acknowledgment of DoD support.**
- 3) $\text{TNF}\alpha$ in epithelial cells is driving an inflammatory reaction in the fibroblast.**
- 4) IL8 from the fibroblast induces $\text{TNF}\alpha$ expression together with epithelia-derived EGF.**
- 5) T-cell recruiting factors are produced by both cancer cells as well as inflammatory infiltrating immune cells.**
- 6) Response to the dynamic cross talk observed in vitro is extremely consistent feature of cancer tissue, with high degree of specificity to cancer over other inflammatory conditions.**

Conclusions:

Deeper understanding of microarray results is achieved via insights gained from in vitro simulation experiments, where the gene-event relation can better be assigned. In that way, microarray forms the bridge between the patient complex condition, and simple model system where enormous mechanistic insight has already been gained. We try to direct this new development to research that may lead to focused novel drug development and diagnostic tools.

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